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Ligand efficiency in axial and radial flow immunoaffinity chromatography of factor IX

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Abstract

Radial flow (RF) columns are attractive for process chromatography primarily because larger throughputs and lower pressure drops are achievable in such columns. Large scale immunoaffinity processes using soft resins can benefit most from this configuration. In this study, we compared immobilized ligand efficiency in axial flow (AF) and RF columns using monoclonal antibody against factor IX as the immobilized ligand and a coagulation factor IX complex as the source material. We examined the effects of flow-rate, total protein loading, feed antigen concentration and direction of flow (centrifugal or centripetal for RF, and downward or upward for AF) on immobilized antibody capacity (measured as mg antigen bound per mg antibody). Our results corroborate earlier work, and suggest that none of the factors, in the ranges examined, significantly altered the efficiency of the monoclonal antibody (MAb) in binding factor IX. We also investigated the efficiency of the immobilized antibody upon reuse and found that, over twenty cycles, there was no significant decrease in antibody efficiency. Our results demonstrated that efficiencies obtainable in AF columns can be achieved in RF columns with the same bed thickness, suggesting that radial dispersion, mass transfer and intraparticular diffusion may not have a significant influence on immunoaffinity chromatography efficiency in RF and AF columns.

1. Introduction

Protein isolation and purification from complex mixtures is becoming increasingly important to the advancement of the biotechnological industry. Immunoaffinity chromatography (IAC) is a specific, reversible purification method utilizing antibodies directed against the protein of choice [1]. IAC has only recently been applied on a large scale using conventional axial flow (AF) chromatography columns [2], and problems documented before [3] include high pressure drops leading to low throughputs and gel compression. Scaling-up from bench to production exacerbates these problems.

A potential solution for AF chromatography problems resides with radial flow (RF) chromatographic column configurations, due to the shorter bed depth [3]. Radial flow was originally developed to handle large gas flow-rates through packed beds with minimal pressure drop [4]. Analytical work has been done on the fluid mechanics [5,6] and the chemical kinetics and dynamics of heterogeneous catalysis in such systems [7]. Analysis and experimentation with RF reactors has been extended to reverse osmosis systems [8,9], as well as hollow fiber cell culture bioreactors [10,11]. CUNO [12] and Sepragen [3] manufacture chromatography sys-

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tems in an RF configuration. Planques et al. [13] used RF membrane affinity chromatography to purify plasminogen with greater than 85% recovery and a 110-fold increase in the specific activity. Other separations of biologicals have also been reported [14].

In the AF column, the cross-sectional area normal to flow is constant; in the RF column, the area normal to flow is variable, increasing for centrifugal flow and decreasing for centripetal flow. This results in a linear velocity that is decelerating (centrifugal flow) or accelerating (centripetal flow). The implications of this is that mass transfer coefficients and radial dispersion cannot be taken as constant [15] in the flow direction. The RF configuration provides a larger flow area and a shorter flow path that permits larger volumetric flow-rates and shorter step times [15] in liquid chromatographic separations.

We have demonstrated [16] lower process times and trans-chromatographic bed pressures for RF compared to AF. In this paper, we compare an AF and RF column of the same total volume (50 ml) and bed height (AF: 2.8 cm; RF: 3.0 cm) using immunoaffinity purification of factor IX (FIX) [17,18] as the experimental system. The effect of changing feed flow-rates, feed antigen concentration and flow direction on antibody efficiency is reported.

2. Materials and methods

2.1. Antibody and resin

The antibody was monoclonal, produced at the American Red Cross (ARC; [19]) and coupled to Sepharose CL2B (Pharmacia, Piscataway, NJ, USA) via CNBr activation. This affinity resin was generously donated by the ARC.

2.2. Starting material

Feed for all the immunoaffinity purification experiments was coagulation FIX, lot 29061202, supplied by the ARC, which is an FIX complex containing 10% (w/w) FIX [20].

2.3. Columns

The AF column was a 4.8 cm diameter glass column (Kontes Glass, Vineland, NJ, USA) that was silanized before use. The column was packed to a depth of 2.8 cm, yielding a total AF column volume of 50 ml. The RF column was purchased from Sepragen (San Leandro, CA, USA) and had a volume of 50 ml. The RF column was packed according to the manufacturer's instructions to a final volume of 50 ml with the affinity resin. Bed depth in the RF column was 3.0 cm.

2.4. Other equipment

Pumping of mobile phase buffers was provided by a Masterflex digital unified drive pump (Cole Parmer, Chicago, IL, USA). Protein flow through from columns was monitored using a Gilson 112 UV–Vis detector (Gilson Medical Electronics, Middleton, WI, USA) and a hard copy of chromatographic output was provided from a Kipp and Zonen BD40 (Delft, Netherlands) single-channel chart recorder. Final absorbance of pooled fractions was measured on a Spectronic 1001+ UV–Vis spectrophotometer (Milton Roy, Downington, PA, USA).

2.5. Chromatography protocol

Columns were first equilibrated with five column volumes (CVs) of equilibration buffer, 10 m*M* magnesium chloride, 100 m*M* sodium chloride, 20 m*M* phosphate, pH 7.0. The starting material, lyophilized coagulation FIX, was reconstituted with distilled water and brought to a final magnesium chloride concentration of 40 m*M*. Feed was then loaded onto the column and then the column was washed with 10 m*M* magnesium chloride, 1 *M* sodium chloride, 20 m*M* phosphate, pH 7.0 buffer until the effluent absorbance reached baseline. FIX was eluted using 20 m*M* sodium citrate, 110 m*M* sodium chloride, pH 6.8. The column was regenerated with 200 m*M* sodium citrate, 2 *M* sodium chloride, pH 7.0 buffer. Column effluent was collected as pools for each step, i.e. load pool,

unadsorbed (unbound material), wash pool, elution pool and regeneration pool and then assayed for total protein. Elution pools were periodically checked using a Gilson HPLC system with a TSK-3000 size-exclusion column for purity, always resulting in a single peak.

2.6. Flow direction variation

RF Column

Feed was pumped into the inner annulus for centrifugal flow or into the outer annular space for centripetal flow. In order to minimize compression during operation, the column was packed under higher than normal operational pressure. After packing, the operation of the column mirrored the AF column's operation in terms of feed, wash and elution.

AF Column

Feed was pumped into the axial flow column, either in a downward flow configuration or in an upward flow configuration.

2.7. Protein assay

Total protein was determined from the absorbance of the sample at 280 nm. The extinction coefficient was assumed to be 1.4. Samples with absorbances greater than 1.0 on direct reading were diluted to bring the absorbance into the range $0.0 < A_{280} < 1.0$.

2.8. Flow-rate variation

Flow-rate in both the AF and RF column experimental sets was varied. Flow-rate ranges for each set of experiments with the different columns ranged from 0.3 to 15 ml/min.

2.9. Feed concentration variation

Concentration of FIX in the feed stream was decreased by increasing the amount of distilled water used to reconstitute the coagulation FIX from the lyophilized vial. Three different reconstitution volumes were used, 5, 10 and 15 ml, resulting in protein concentrations of 6.9, 3.45 and 1.7 mg/ml, respectively.

3. Results and discussion

To evaluate affinity column performance, we compared the efficiency of the antibody, defined as the mass of antigen that is bound per unit mass of immobilized antibody. In order to calculate a *theoretical* maximum capacity for the immobilized antibody, we make the assumption that *if* an antibody is immobilized at the Fc portion, it will be fully active and capable of binding two antigen molecules. Naturally, CNBr activation of Sepharose is not suitable for immobilization through the Fc region; carbohydrate moiety coupling will provide a better condition for Fc immobilization [21,22]. However, for the purpose of our column antibody efficiency comparison, such a *theoretical* maximum provides a basis, and with FIX as the antigen (molecular mass ca. 50 000) a single immobilized completely active antibody (molecular mass ca. 150 000) would theoretically be able to bind two FIX molecules. On a mass basis, this theoretical capacity would be approximately 0.66 mg FIX/mg of antibody [18,23]. With this as a criterion, it is possible to evaluate the bulk performance of immobilized antibody in a particular process by estimating the mass of antigen captured per unit mass of immobilized antibody. This is possible if the amount of immobilized antibody is known and if the total amount of antigen bound is calculated from the difference between the mass of antigen loaded and the mass of antigen that passed through the affinity column in the unadsorbed pool, and also by calculating the amount of FIX protein that is recovered in the elution and regeneration steps of the process.

In our work, we report the effect of varying parameters (feed flow-rate and antigen concentration) and flow direction (centripetal/centrifugal RF or upward/downward AF) on the capacity of the antibody.

In Fig. 1, the results of varying the flow-rate in AF and RF columns is shown. Fig. 1a compares

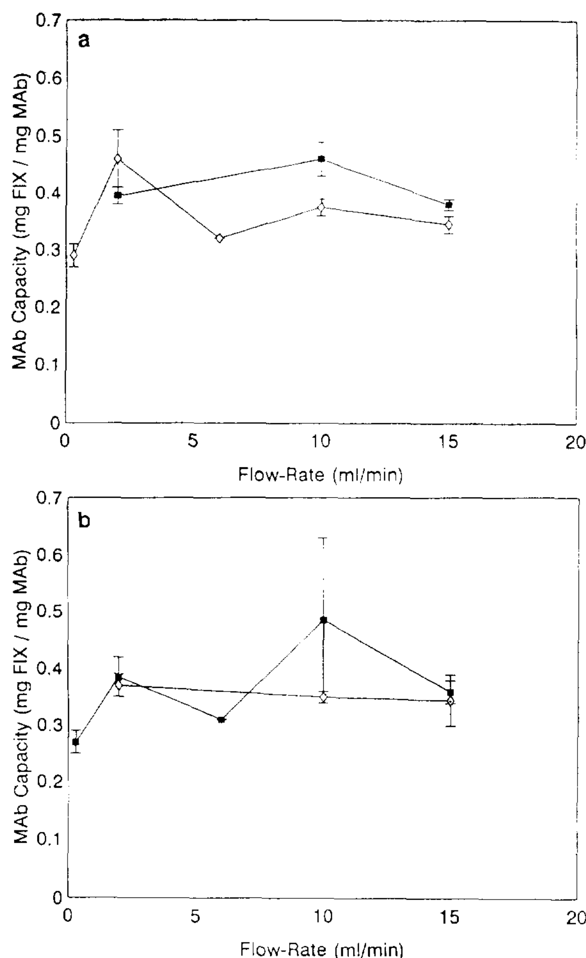

Fig. 1. Effect of flow-rate on antibody capacity. (a) Centrifugal radial flow (■) and downward axial flow (◇); (b) centripetal radial flow (■) and upward axial flow (◇). Data are reported as the mean ligand efficiency obtained for each flow-rate ± 1 S.D.

the antibody capacity between centrifugal RF and the equivalent downward AF. As the flow-rate increases, there is no statistically significant increase or decrease in antibody capacity for either of the flow configurations. These data are in agreement with our previous work [18] which showed no dependence of antibody capacity on flow-rate in an AF chromatography system. The ranges of flow-rate examined were similar. In Fig. 1b, antibody capacity as a function of flow-rate is shown for the case of both flow configurations with reversed flow, i.e. centripetal for the RF case and upward flow for the AF situation. Once again, we see that there is no statistical difference in antibody capacity for increasing flow-rate.

Fig. 2 shows the effect of increasing feed protein concentration on the capacity of antibody. The data are presented in a similar manner as in Fig. 1. Thus, we see centrifugal RF and downward AF in Fig. 2a, and in Fig. 2b the results for the reversed flow direction, centripetal RF and upward AF, are shown. The data in Fig. 2 suggests that there is no significant dependence of antibody capacity on feed protein concentration. The data in Fig. 2a do show a slight increase in the antibody capacity for the

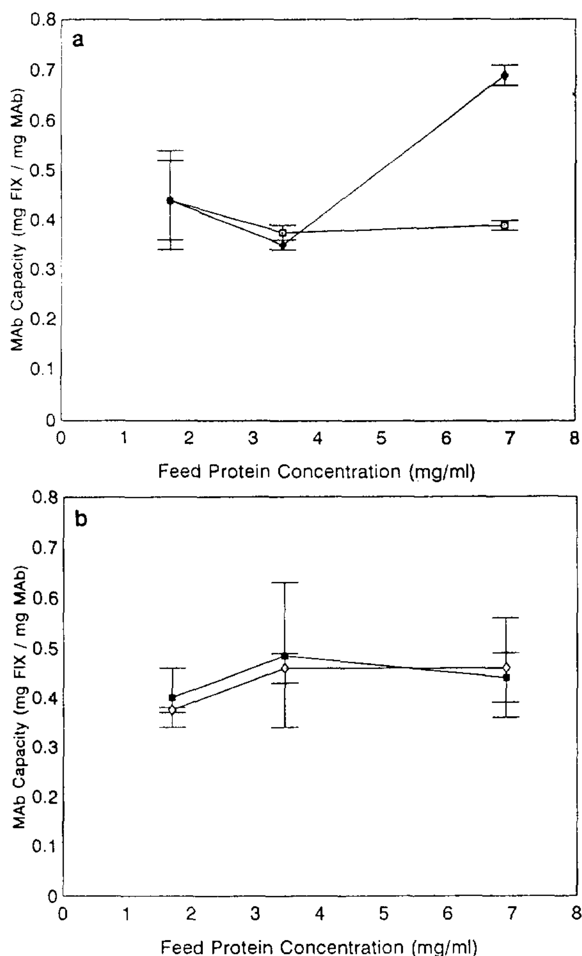

Fig. 2. Effect of feed protein concentration on antibody capacity. (a) Centrifugal radial flow (□) and downward axial flow (◆); (b) centripetal radial flow (■) and upward axial flow (◇). Data are reported as in Fig. 2.

centrifugal radial configuration. There is a slight increase for the axial configuration as well, although not as large as the RF data. Neither of the increases appear to be statistically significant, and it appears that over the ranges of inlet protein concentration studied, the antibody efficiency remained similar in both AF and RF.

We have suggested with earlier work [23] that a strong dependence on feed protein concentration and flow-rate would indicate that diffusional resistances for antigen binding to antibody would be high. In the absence of these strong dependences, as this and our previous data have shown, it is likely that diffusional resistance to antigen binding is low. We have previously argued [23], using the work of Carleysmith et al. [24], that the reason for this might lie in the depth to which antibody is immobilized. If antibody is only immobilized on a thin outer skin —say 10% of the radial co-ordinate, or about 8 μm— of the resin bead, it is unlikely that antigen will encounter diffusional resistances. This inference is supported by the data for the effect of flow-rate on antibody capacity. If diffusion was a significant resistance to antigen penetration, and hence antibody capacity, decreasing the flow-rate, or increasing the antigen feed concentration, would increase antibody capacity, a result not borne out by our data.

Gu et al. [15] have theoretically investigated RF chromatography and their results show that models for RF and AF columns become the same when radial dispersion and intraparticular diffusion are neglected and when the mass transfer coefficients are treated as constants. They also showed that under these conditions, centrifugal and centripetal flow differences disappeared. This and our experimental results suggest that, for IAC under our experimental conditions, radial dispersion, mass transfer and intraparticular diffusion may be neglected if the affinity ligand is only immobilized on a thin outer skin of the chromatography resin bead matrix.

Currently in our laboratory, we are staining and sectioning resin beads that have had antibody immobilized on them. The anticipated data should show the depth of penetration of antibody into the resin bead as a function of immobilization time, chemistry, bead porosity and other parameters. Preliminary data suggest that the penetration depth of the antibody into an activated Sepharose resin, when using CNBr-activated Sepharose as the affinity support, is of the order of 10% of the bead radius [25]. In addition to studies on the distribution of antibody, investigations of the distribution of antigen are also being initiated. Results from these investigations should shed light on the distribution and movement of ligand and ligate within an affinity support matrix, and the impact these have on the efficiency of the ligand.

Future efforts on the comparison of AF and RF chromatography will include studies on scaled versions of industrial columns. Since the bed heights of industrial-process RF and AF columns are very different, experiments are planned which will investigate columns of similar volume with different bed heights. Finally, the efficiency of ligand immobilized on newer, more structurally stable resins should be investigated as these resins may enable higher throughputs in immunoaffinity AF chromatography configurations.

4. Conclusions

A better understanding of RF IAC will facilitate its incorporation into the bioprocess industry. The data presented in this study show that antibody capacities equivalent to those of a conventional AF column are obtainable in an RF configuration. These results suggest that radial dispersion, mass transfer and intraparticular diffusion may not have a significant impact on IAC.

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